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CD8$^+$ T cells characterize early smoking-related airway pathology in patients with asthma

A. Janneke Ravensberg [a,*], Annelies M. Slats [a], Sandra van Wetering [a], Kirsten Janssen [a], Simone van Wijngaarden [a], Ronald de Jeu [a], Klaus F. Rabe [a,b], Peter J. Sterk [a,c], Pieter S. Hiemstra [a]

[a] *Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Centre of Pneumonology, Grosshansdorf Hospital, Grosshansdorf, Germany*
[c] *Department of Respiratory Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

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KEYWORDS
Asthma;
Smoking;
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Summary
Background: Smoking in asthma occurs frequently and is associated with increased symptom severity, an impaired response to corticosteroids, and accelerated lung function decline. Airway pathology in smoking asthmatics is characterized by neutrophilia and epithelial changes such as goblet cell hyperplasia and increased proliferation. Bronchial CD8$^+$ T cells are implicated in lung function decline in asthma and COPD. We hypothesized that smoking modifies airway inflammation in asthma by increasing the number of CD8$^+$ T cells at an early stage.
Objectives & methods: To study effects of smoking on airway pathology in bronchial biopsies from atopic patients with controlled intermittent or mild persistent asthma (12 smokers, 9.7 py and 11 never-smokers, 0.0 py; 20–50 yrs; $FEV_1 > 70\%$ predicted; $PC_{20}MCh < 8$ mg/mL, no ICS) using immunohistochemistry.
Results: Smoking asthmatics showed higher numbers of bronchial CD8$^+$ T cells (55.8 vs 23.9 cells/0.1 mm^2; $p = 0.001$) and CD68$^+$ macrophages (7.5 vs 4.6 cells/0.1 mm^2, $p = 0.012$), and a lower CD4$^+$/CD8$^+$ cell ratio (0.16 vs 0.40; $p = 0.007$) compared with non-smoking asthmatics, but no difference in neutrophils. Furthermore, the % intact epithelium was higher in smoking asthmatics (49.3 vs 23.3, $p = 0.001$).
Conclusion: Smoking asthmatics with a limited smoking history show a distinct pattern of airway pathology characterized by a bronchial infiltrate of CD8$^+$ T cells and CD68$^+$

* Corresponding author. Department of Pulmonary Rehabilitation, Rijnlands Rehabilitation Center, P.O. Box 176, 2300 AD Leiden, The Netherlands. Tel.: +31 71 5195300; fax: +31 71 5195153.
E-mail address: jra@rrc.nl (A.J. Ravensberg).

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macrophages, and epithelial remodelling resembling COPD-like features. This raises the hypothesis that early presence of $CD8^+$ T cells contributes to disease progression in smoking asthmatics.

Introduction

Smoking occurs frequently in patients with asthma. Recent surveys on smoking prevalence report 21—26% current smokers in populations of patients with asthma.[1,2] Detrimental effects of active smoking in asthma include worse asthma control,[3,4] an impaired response to corticosteroids[5,6] and accelerated lung function decline.[7—9]

The mechanisms by which cigarette smoking contributes to disease severity in asthma are incompletely understood, but it has been suggested that cigarette smoking may change inflammation and airway remodelling in asthma to become more similar to that in COPD.[10] Cigarette smoking by itself is associated with airway inflammation and features of airway remodelling including increased epithelial proliferation,[11] squamous cell metaplasia,[12] goblet cell hyperplasia, smooth muscle hypertrophy, and increases in bronchial glands mass.[13,14] In asthma, airway inflammation and airway remodelling are thought to cause impairment in lung function. Allergic inflammation in asthma is characterized by increased numbers of $CD4^+$ T cells, eosinophils and mast cells, and has been associated with changes in airway epithelium such as increased mucin production. Furthermore, $CD8^+$ T lymphocytes have been implicated in lung function decline in asthma.[15] Smokers with chronic obstructive pulmonary disease (COPD) show a distinct cellular infiltrate characterized by $CD8^+$ T lymphocytes which has been suggested to be related to airflow limitation.[16,17] It could be postulated that these cells play a role in the observed progressive lung function decline in smoking asthmatics. Thus far, it has been reported that smoking asthmatics show neutrophilia and increased expression and production of IL-8 in sputum samples and bronchial biopsies, whereas eosinophilia is less prominent compared with non-smoking asthmatics.[18—21] Furthermore, epithelial changes such as goblet cell hyperplasia, squamous cell metaplasia and higher proliferation rate have been reported.[19,21] To date, the influence of smoking on $CD8^+$ T lymphocyte infiltration in the bronchial mucosa of smoking asthmatics has not been studied. In view of potential development of an overlap syndrome between asthma and COPD,[22] the influence of smoking on inflammation in asthma *per se* should preferably be examined at early stages of smoking history.

We hypothesized that smoking modifies airway pathology in asthma at an early stage resulting in an inflammatory cellular infiltrate in the bronchial mucosa characterized by increased numbers of $CD8^+$ T cells. To this end we analysed bronchial biopsies from steroid-naïve non-smoking and smoking patients with intermittent and mild persistent asthma. Smokers with asthma were characterized by a limited smoking history and duration.

Methods

Subjects and study design

Two groups of patients with intermittent and mild persistent asthma[23] were included in this cross-sectional study. Bronchial biopsy samples, lung function measurements and clinical data from twelve asthmatics who were never-smokers were available from a previous investigation.[24] Twelve smoking asthmatics were newly recruited during an overlapping time period. At screening, medical history was taken and lung function,[25] airway hyperresponsiveness[26] and allergy to 10 common air-borne allergens[27] were determined. During the second visit reversibility was determined and a bronchoscopy was performed to obtain bronchial biopsies. To minimize variation in laboratory readings between the two groups, identical and standardized study procedures were used which were performed by the same laboratory technicians.

Lung function expressed as forced expiratory volume in 1 s (FEV_1) was >70% predicted post-bronchodilator.[25] All patients were hyperresponsive to methacholine (provocative concentration causing a 20% fall of FEV_1 (PC_{20}) < 8 mg/ml)[26] and atopic. They were well-controlled with bronchodilators only, and had not used oral or inhaled corticosteroids 3 months prior to and during the study. Smoking asthmatics were current smokers with 7.5—25 py. The institutional review board for human studies approved the protocol and subjects gave written informed consent.

Bronchoscopy, immunohistochemistry and image analysis

Smokers were requested to refrain from smoking ≥ 1 h before bronchoscopy was performed according to a standardized protocol.[28] Three biopsies were taken at (sub) segmental level, fixed in formaldehyde and embedded in paraffin. For both groups haematoxylin-eosin stained sections were used to select the two morphological best biopsies per patient for analysis.[29] The observer was blinded with regard to the patient's number and clinical status during selection, processing and analysis of the biopsy samples.

Immunohistochemistry was performed simultaneously for both groups on newly cut sections using mouse monoclonal antibodies directed against CD3, CD4, CD8 (T lymphocytes), neutrophil elastase (NE), CD68 (macrophages), EG2 (eosinophils) and AA1 (mast cells) to identify the number of inflammatory cells per 0.1 mm^2 using digital analysis in a 125 μm deep zone beneath the basement membrane (BM) from which glands and airway smooth muscle were excluded. All cell counts were performed

twice by an experienced observer. Intraclass correlation coefficients showed high consistency between repeated measurements (range: 0.996–0.999).

Mucin producing cells were identified using Periodic acid-Schiff/Alcian blue (PAS/AB) and densitometry analysis to determine the percentage of positively stained intact epithelium. For evaluation of the epithelium the length of the BM was traced of all intact non-squamous metaplastic epithelium, intact squamous metaplastic epithelium, and damaged epithelium.[30] To determine epithelial proliferation the number of Ki67 positively stained cells was counted in intact epithelium (numbers per length of BM).

Statistical analysis

Data are reported as mean values and standard deviations (SD) or median with interquartile range (IQR). Between-group differences were analysed by 2-tailed unpaired *t*-tests for patient characteristics and Mann–Whitney tests for data from biopsy samples. Spearman's rank correlation coefficients were determined to explore associations between functional parameters and data from bronchial biopsies. *P* values < 0.05 were considered to indicate statistical significance.

Results

Patient characteristics

After evaluation of the bronchial biopsies, one patient from the group of non-smoking asthmatics was excluded from analysis because of haemorrhagic biopsy samples. The characteristics of the 11 non-smoking and 12 smoking patients with intermittent and mild persistent asthma are given in Table 1. Smoking asthmatics were current smokers with a median smoking duration of 11 years and 9.7 pack-years. They were slightly older than the non-smoking asthmatics (median age 27 vs 22 years respectively; $p < 0.05$). There were no significant differences between the groups with respect to asthma duration, lung function, reversibility or airway hyperresponsiveness. All patients were atopic to one or more of 10 common air-borne allergens.

Bronchial inflammation

Smoking asthmatics had significantly more $CD8^+$ T lymphocytes (Figs. 1 and 2) and $CD68^+$ macrophages, and less eosinophils in the bronchial mucosa compared with non-smoking asthmatics (Fig. 2). The ratio of $CD4^+/CD8^+$ T cells was significantly lower in smoking asthmatics. There were no differences between groups in the numbers of $CD3^+$ and $CD4^+$ T lymphocytes, mast cells and neutrophils (Table 2). The median area of lamina propria analysed was 0.32 (0.18–0.52) mm^2.

Features of airway epithelium

Smoking asthmatics showed a significantly higher percentage of intact epithelium compared with non-smoking asthmatics [52.0 (37.2–67.3) vs [23.3 (18.3–33.7); $p = 0.001$] (Fig. 3), whereas the percentage of PAS/AB^+ area [12.0 (5.2–18.2) vs 5.8 (4.0–12.7), respectively] and the number of $Ki67^+$ proliferating cells [9.2 (4.4–24.7) vs 5.1 (0.83–12.5), respectively] did not differ between groups. The median analysed BM length per biopsy was 2.1 (1.1–4.8) mm. Squamous cell metaplasia was observed in two smoking asthmatics but not in non-smoking asthmatics (Fig. 4).

Relation between smoking, lung function, bronchial inflammation and features of airway epithelium

In smoking asthmatics no significant correlations were found between smoking duration or pack-years, and lung function (FEV_1 % predicted or FEV_1/FVC) pre or post bronchodilator.

The numbers of inflammatory cells in the lamina propria were not significantly associated with pack-years or smoking duration in smoking asthmatics. There was only a trend towards a significant relation between the number of pack-

Table 1 Patient characteristics.

	Non-smoking asthmatics	Smoking asthmatics
N	11	12
Gender [M/F]	4/7	4/8
Age [years]	22 (21–24)	27 (24–40)[a]
FEV_1 post [%predicted]	103.9 ± 10.8	98.0 ± 11.3
FEV_1/FVC post	0.86 ± 0.07	0.80 ± 0.10
PC_{20} [mg/ml]	1.13 ± 1.48	1.91 ± 1.76
Reversibility FEV_1 [%]	8.8 ± 3.9	8.2 ± 5.1
Asthma duration [years]	14.1 ± 7.7	18.6 ± 10.0
Atopy [nr of atopic patients]	11	12
Smoking history [pack-years]	0.0 (0.0–0.0)	9.7 (8.3–17.0)[a]
Smoking duration [years]	0.0 (0.0–0.0)	11.0 (10.3–25.8)[a]
Cigarette consumption [cigarettes/day]	0.0 (0.0–0.0)	14 (11–18)[a]

PC_{20} methacholine is represented as geometric mean (SD in doubling dose). Other data are represented as mean ± SD or median (IQR).
[a] $p < 0.05$ smoking vs non-smoking asthmatics.

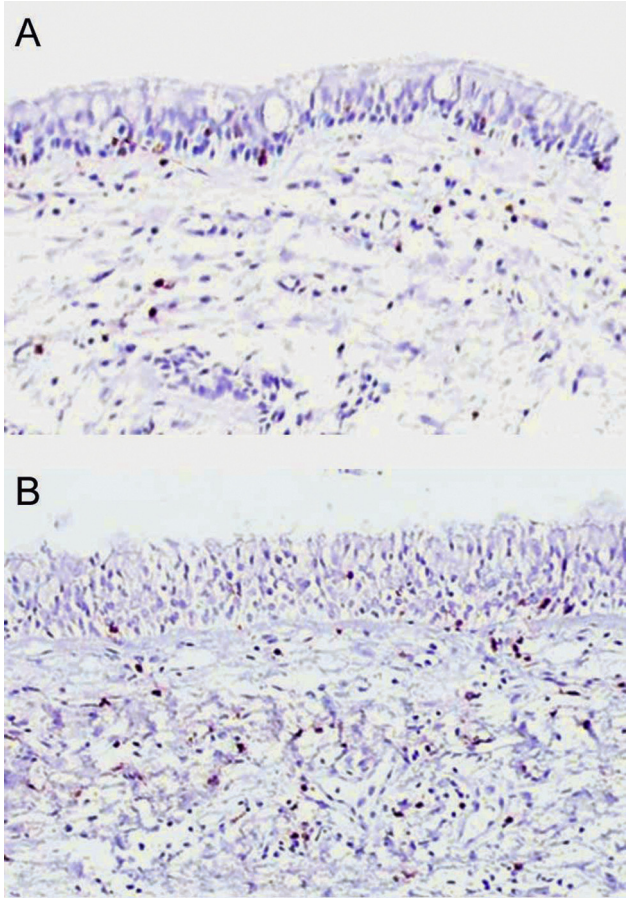

Figure 1 $CD8^+$ T lymphocyte infiltration observed in bronchial biopsies obtained from a non-smoking [A] and smoking [B] asthmatic patient. Original magnification: ×200.

years and $CD68^+$ macrophages ($r = 0.57$; $p = 0.053$) in this group. When smoking and non-smoking asthmatics were analysed together, pack-years and smoking duration were significantly associated with the number of $CD68^+$ macrophages ($r = 0.57$; $p = 0.004$ and $r = 0.57$; $p = 0.008$, respectively) and $CD8^+$ T cells ($r = 0.61$; $p = 0.002$ for both parameters), whereas inverse relationships with the ratio of $CD4^+/CD8^+$ T cells were also found ($r = -0.43$; $p = 0.043$ and $r = -0.44$; $p = 0.035$, respectively). In smoking asthmatics, lung function was not related to the number of $CD8^+$ T cells.

Pack-years and smoking duration were not significantly associated with features of airway epithelium. When both groups were analysed together the numbers of $CD68^+$ macrophages and $CD8^+$ T cells and $CD4^+/CD8^+$ cell ratio were significantly associated with the percentage of intact epithelium ($r = 0.47$; $p = 0.023$, $r = 0.60$; $p = 0.003$, and $r = -0.47$; $p = 0.023$, respectively).

Discussion

This study evaluated the effects of smoking on bronchial inflammation and airway epithelium in steroid-naïve patients with mild intermittent and persistent asthma. Smokers with asthma with a limited smoking history show a distinct pattern of bronchial inflammation characterized by higher numbers of $CD8^+$ T lymphocytes and $CD68^+$ macrophages and lower numbers of eosinophils whereas the airway epithelium showed less damage compared with never-smokers with asthma. These features represent early smoking-induced airway pathology and may contribute to disease progression in asthma.

In line with our hypothesis, we demonstrated that increased numbers of $CD8^+$ T lymphocytes and a decreased $CD4^+/CD8^+$ T cell ratio characterize the cellular infiltrate in the lamina propria in smokers with asthma compared to never-smokers with asthma. Increased numbers of $CD8^+$ T lymphocytes are also found in peripheral and central airways of smokers with COPD compared to smokers without airflow limitation. An association between $CD8^+$ T lymphocytes and lung function has been described in a combined group of smokers with and without airflow limitation.[16,17,31] Our patients had relatively few pack-years which could explain the fact that we did not observe such a relation. It could be argued that the number of subjects included in our study was limited. However, a sample size of 10 patients per group was estimated to detect a two-fold difference in $CD8^+$ T cells with a power of 90%, and indeed we found a significant difference. Since the focus of the current study was to determine whether smoking induces changes in airway pathology in asthma, we did not include healthy subjects. Therefore, we cannot answer the question whether asthma is a prerequisite for smoking-induced bronchial infiltration by $CD8^+$ T lymphocytes in our group of patients. Studies comparing bronchial biopsies obtained from smokers with normal lung function and never-smokers showed no difference in the number of $CD8^+$ T lymphocytes.[16,17,31,32] However, these groups were not comparable with our cohort of patients with respect to age and smoking history. Thus, we can only speculate that existing lung pathology is required for increased $CD8^+$ T cell infiltration in the bronchial mucosa.

What mechanisms cause $CD8^+$ T lymphocytes to accumulate in the lung and what could be the consequence? First of all, endogenous stimuli such as modified self-antigens induced by xenobiotic compounds and/or free radicals in cigarette smoke could contribute to recruitment of these cells. Also exogenous factors that elicit an immune response could be involved such as viruses. Smokers have a higher frequency of viral infections than non-smokers.[33,34] Asthma patients who smoke show reduced numbers of $CD83^+$ mature dendritic cells and B lymphocytes compared with never-smokers with asthma[35] and therefore may be more prone to develop lower respiratory tract infections. Viral infections can induce antigen-specific $CD8^+$ T lymphocytes persisting in the lungs for many months.[36] Persistence and activation of $CD8^+$ lymphocytes could contribute to airway and remodelling thereby adding to the development of airflow impairment.[37] In fact, lower respiratory tract infections in smokers with mild COPD and exacerbations in asthma have been associated with decline in lung function.[38,39] Moreover, $CD8^+$ T lymphocytes have been shown to be related to the annual decline in FEV_1 in patients with asthma.[15] Our findings and the fact that smoking has been associated with an accelerated decline in lung function in asthma[7–9] may suggest that $CD8^+$ T lymphocytes could have a significant role in the smoking-induced airway pathology and clinical course of asthma.

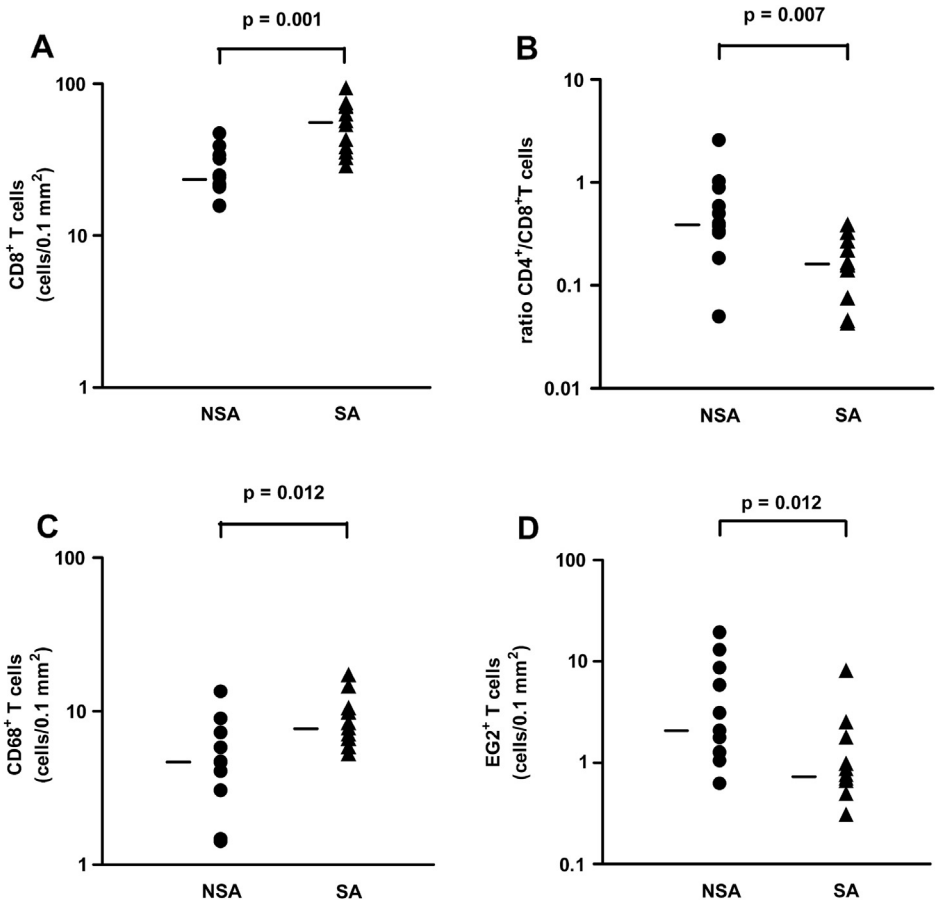

Figure 2 $CD8^+$ T cells [A], ratio of $CD4^+/CD8^+$ T cells [B], $CD68^+$ cells [C], and $EG2^+$ cells [D] in bronchial mucosa from non-smoking and smoking asthmatics (NSA and SA respectively). Cell counts are expressed as numbers of cells per 0.1 mm^2; medians are depicted as a horizontal line.

The inflammatory profile in our population of smokers is also characterized by higher numbers of $CD68^+$ macrophages and lower numbers of eosinophils compared with non-smokers, whereas the number of neutrophils did not differ between groups. Reduced eosinophilia in smokers with asthma has been reported previously in both sputum[5,20] and bronchial tissue,[21] but has not been confirmed in all studies investigating effects of smoking on bronchial inflammation in asthma.[18,19] We found no significant difference in bronchial neutrophilia between groups, whereas others have reported higher numbers of

Table 2 Bronchial inflammation in non-smoking and smoking patients with asthma.

	Non-smoking asthmatics	Smoking asthmatics	*P* value
$CD3^+$ cells	61.0 (35.0—89.1)	55.3 (50.9—70.9)	0.951
$CD4^+$ cells	12.4 (5.9—18.7)	8.9 (4.7—13.4)	0.176
$CD8^+$ cells	23.9 (20.7—33.6)	55.8 (36.4—72.0)	0.001
Ratio $CD4^+/CD8^+$ cells	0.40 (0.32—0.88)	0.16 (0.09—0.32)	0.007
$CD68^+$ cells	4.6 (1.5—7.2)	7.5 (6.1—10.4)	0.012
NE^+ cells	2.1 (0.6—2.8)	2.3 (0.3—3.8)	0.854
$EG2^+$ cells	2.1 (1.2—8.6)	0.7 (0.5—1.6)	0.012
$AA1^+$ cells	12.7 (9.9—16.5)	6.6 (1.9—13.9)	0.124

Numbers of cells are expressed as median (IQR) per 0.1 mm^2 lamina propria.

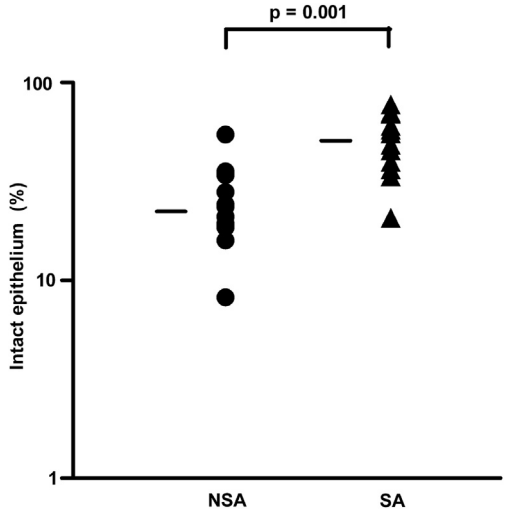

Figure 3 Percentage intact epithelium in bronchial biopsies obtained from non-smoking (NSA) and smoking (SA) asthmatic patients. Medians are depicted as a horizontal line.

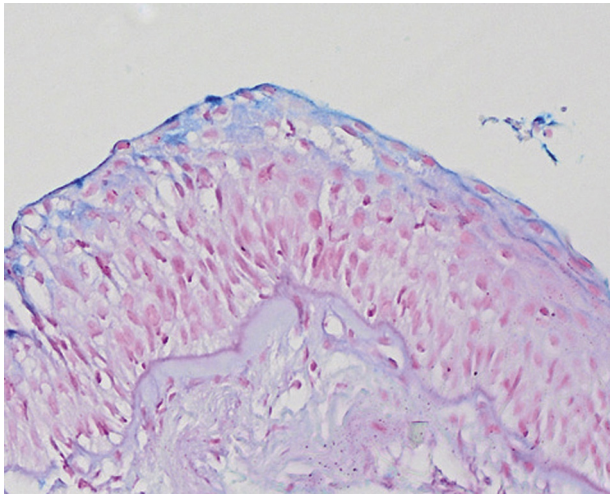

Figure 4 Squamous cell metaplasia in a bronchial biopsy obtained from a smoking asthmatic patient. Original magnification: ×200.

neutrophils in both sputum[18,20] and bronchial tissue,[19] and increased expression of IL-8 in airway epithelium[19] in smokers with asthma. It could be postulated that neutrophilic inflammation is related to the intensity and duration of smoking since our smoking asthmatics were younger, consumed fewer cigarettes and had less pack-years than the subjects of the above mentioned studies. Indeed, in a population of asthmatics with similar characteristics to ours no smoking-related difference in sputum neutrophilia was detected.[6] It has been suggested that young asthmatic smokers may develop early COPD-like features based on the observation that they exhibit airway neutrophilia, lung hyperinflation, lower lung diffusion capacity, increased baseline airway resistance and emphysematous changes on HRCT.[18] The inflammatory profile we found in our group of asthmatic smokers may support this concept.

Although both asthma[40] and smoking are associated independently with goblet cell hyperplasia[13,14] we did not detect an increased PAS/AB$^+$ area within the airway epithelium in smoking asthmatics. Furthermore we found no difference between the numbers of proliferating Ki67$^+$ epithelial cells. These results may have been due to a lack of power since three non-smoking asthmatics whose biopsy samples showed damaged epithelium only were excluded from statistical analysis. In fact, increased proliferation of epithelial cells and goblet cell hyperplasia has been reported in smoking asthmatics compared with non-smoking asthmatics and was associated with increased epithelial thickness.[21] We did find that the airway epithelium in smoking asthmatics showed significantly less damage than that in the non-smoking asthmatics. Since the biopsy samples from both groups were collected and processed using identical procedures, it is unlikely that the difference in epithelial loss between groups is an artifact due to sampling procedures. Several studies support the hypotheses of increased epithelial fragility in asthma[41–43] and an aberrant response to epithelial injury in asthma.[44–46] Smoking on the other hand, is associated with increased proliferation of epithelial cells and wound repair.[47,48] It could be speculated that such an adaptive response in smoking asthmatics could serve as a defence mechanism protecting the airway epithelium resulting in less injury compared with non-smoking asthmatics or — in the long term- could result in aberrant epithelial repair contributing to altered epithelial function in these patients. Alternatively, increased epithelial proliferation may be a specific phenotype *per se* that facilitates the persistence of smoking in asthma.

Our study shows that smoking induces changes in airway pathology in asthmatics with a normal lung function at an early stage, i.e. in those with a limited smoking history and duration. The inflammatory profile characterized by increased numbers of CD8$^+$ T lymphocytes and CD68$^+$ macrophages may be related to the accelerated lung function decline found in smoking asthmatics and might may represent early COPD-like changes which could have consequences for clinical outcomes and asthma treatment response.[10] This underlines the great importance of smoking abstinence and cessation in asthmatics. Thus far, smoking cessation has been shown to be successful in improving lung function in asthma.[49]

In conclusion, our study results indicate that active smoking in asthma causes a distinct pattern of bronchial inflammation characterized by infiltration of CD8$^+$ T lymphocytes and CD68$^+$ macrophages, and epithelial remodelling resembling COPD-like features. Further research is warranted to investigate whether the early presence of this particular type of inflammation contributes to disease progression in smoking asthmatics.

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Conflict of interest statement

The authors have no financial or other potential conflicts of interest to disclose.

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